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The Lysozyme-Induced Peptidoglycan *N*-Acetylglucosamine Deacetylase PgdA (EF1843) Is Required for *Enterococcus faecalis* Virulence

Abdellah Benachour,[a] Rabia Ladjouzi,[a] André Le Jeune,[a] Laurent Hébert,[a] Simon Thorpe,[b] Pascal Courtin,[c,d] Marie-Pierre Chapot-Chartier,[c,d] Tomasz K. Prajsnar,[e,f] Simon J. Foster,[e,f] and Stéphane Mesnage[e,f]

Université de Caen Basse-Normandie, EA 4655 U2RM, Caen, France[a]; Department of Chemistry, University of Sheffield, Brookhill, Sheffield, United Kingdom[b]; INRA, UMR1319 Micalis, Jouy-en-Josas, France[c]; AgroParisTech, UMR Micalis, Jouy-en-Josas, France[d]; Krebs Institute, University of Sheffield, Western Bank, Sheffield, United Kingdom[e]; and Department of Molecular Biology and Biotechnology, University of Sheffield, Western Bank, Sheffield, United Kingdom[f]

Lysozyme is a key component of the innate immune response in humans that provides a first line of defense against microbes. The bactericidal effect of lysozyme relies both on the cell wall lytic activity of this enzyme and on a cationic antimicrobial peptide activity that leads to membrane permeabilization. Among Gram-positive bacteria, the opportunistic pathogen *Enterococcus faecalis* has been shown to be extremely resistant to lysozyme. This unusual resistance is explained partly by peptidoglycan *O*-acetylation, which inhibits the enzymatic activity of lysozyme, and partly by D-alanylation of teichoic acids, which is likely to inhibit binding of lysozyme to the bacterial cell wall. Surprisingly, combined mutations abolishing both peptidoglycan *O*-acetylation and teichoic acid alanylation are not sufficient to confer lysozyme susceptibility. In this work, we identify another mechanism involved in *E. faecalis* lysozyme resistance. We show that exposure to lysozyme triggers the expression of EF1843, a protein that is not detected under normal growth conditions. Analysis of peptidoglycan structure from strains with *EF1843* loss- and gain-of-function mutations, together with *in vitro* assays using recombinant protein, showed that EF1843 is a peptidoglycan *N*-acetylglucosamine deacetylase. EF1843-mediated peptidoglycan deacetylation was shown to contribute to lysozyme resistance by inhibiting both lysozyme enzymatic activity and, to a lesser extent, lysozyme cationic antimicrobial activity. Finally, *EF1843* mutation was shown to reduce the ability of *E. faecalis* to cause lethality in the *Galleria mellonella* infection model. Taken together, our results reveal that peptidoglycan deacetylation is a component of the arsenal that enables *E. faecalis* to thrive inside mammalian hosts, as both a commensal and a pathogen.

Enterococci are Gram-positive commensal bacteria commonly found in the gastrointestinal and vaginal tracts and in the oral cavity of humans and other mammals. Over the last decades, *Enterococcus faecalis* has emerged as a leading cause of life-threatening nosocomial infections (23). Due to its large range of intrinsic and acquired antibiotic resistances, *E. faecalis* infections can be difficult to treat and are associated with substantial health care costs. In addition to the high capacity of *E. faecalis* to resist harsh conditions and a wide range of stresses (19), genome-wide analyses of clinical isolates suggested that the genome plasticity of this bacterium also contributes to its ability to survive both as a commensal and as an opportunistic pathogen (33, 37). One of the properties enabling *E. faecalis* to survive within the mammalian host is an extreme resistance to lysozyme, one of the most important and widespread components of innate immunity (9). Lysozyme is found in a wide range of body fluids (e.g., tears, saliva, and urine) and tissues (e.g., respiratory and intestinal tract tissues) and is produced by neutrophils and macrophages (7, 13, 17, 29). The bactericidal activity of lysozyme is due both to the cell wall lytic activity of this enzyme [cleavage of β-(1,4)-glycosidic bonds between *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc) residues in peptidoglycan] and to a nonenzymatic mechanism involving its cationic antimicrobial peptide (CAMP) properties that lead to membrane permeabilization (25, 27). To counteract both modes of action of lysozyme, Gram-positive bacteria have developed two major mechanisms: (i) modification of the peptidoglycan structure through peptidoglycan GlcNAc deacetylation (6, 43) or MurNAc *O*-acetylation (4, 10, 24) and (ii) modification of the negative net charge of the bacterial cell envelope to decrease binding of cationic lysozyme to the cell. The latter mechanism is mediated by *dlt* genes, adding positively charged D-alanine residues on both teichoic and lipoteichoic acids (24, 34), or the multiple peptide resistance factor MprF, adding L-lysine on phosphatidylglycerol (16).

Previous studies have shown that *E. faecalis* is highly resistant to lysozyme, with a MIC above 50 mg/ml. This extreme resistance has been partly explained by an additive effect of both peptidoglycan *O*-acetylation and cell wall D-alanylation (24, 30). It was also shown that full lysozyme resistance requires a signaling cascade involving the extracytoplasmic function (ECF) sigma factor SigV. Previous studies revealed that the *E. faecalis* genome encodes a putative peptidoglycan deacetylase (EF1843). Although deletion of EF1843 was associated with a decreased persistence in mouse peritoneal macrophages, no phenotype associated with this deletion could be shown (24). In this work, we identified the mechanism by which *sigV* signaling contributes to ly-

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Address correspondence to Abdellah Benachour, abdellah.benachour@unicaen.fr, or Stéphane Mesnage, s.mesnage@sheffield.ac.uk.
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sozyme resistance. We show that exposure to lysozyme triggers SigV-dependent expression of the peptidoglycan GlcNAc deacetylase EF1843 (PgdA) in *E. faecalis* JH2-2. PgdA-mediated peptidoglycan deacetylation is shown to contribute not only to lysozyme resistance but also to virulence of *E. faecalis* in a *Galleria mellonella* insect model.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. *E. faecalis* was grown at 37°C without shaking in M17 medium supplemented with 0.5% glucose (GM17) (38) or in brain heart infusion (BHI). *Escherichia coli* was grown with vigorous shaking at 37°C in Luria-Bertani (LB) medium (36). When required, erythromycin or ampicillin was added for *E. faecalis* or *E. coli* cultures, respectively.

DNA manipulations. General molecular methods, molecular cloning, and other standard techniques were performed essentially as described in reference 36. Electrocompetent cells of *E. coli* or *E. faecalis* (11) were transformed by electroporation using a Gene Pulser apparatus (Bio-Rad Laboratories). Plasmids and PCR products were purified using Qiagen kits (Qiagen, Valencia, CA).

Plasmid construction for protein expression and gene deletion. PgdA (301 residues) has a putative N-terminal signal peptide of 41 amino acids (24). Recombinant PgdA corresponding to the extracellular portion of the protein was overexpressed in *E. coli* to raise specific antibodies. pQE1843, encoding a polypeptide corresponding to residues 44 to 301 of PgdA, was constructed as follows. The DNA fragment encoding PgdA was PCR amplified with primers LH20 and Pgd19 (see Table S1 in the supplemental material) using *E. faecalis* JH2-2 genomic DNA as a template. The resulting PCR product was digested by BamHI and PstI and cloned into the pQE31 vector (Qiagen) (see Table S1). The construction of pgdMAD, a pMAD derivative with inactivation of the gene encoding PgdA, was done as previously described (24). The following primers, listed in Table S1, were routinely used to check gene deletions: Pgd12 and Pgd19 for *EF1843*, LH41 and LH43 for *EF0783*, Dlt16 and Dlt17 for *EF2749*, and Sig7 and Sig8 for *EF3180*.

Complementation of the *E. faecalis* Δ*pgdA* mutant was obtained by cloning the cognate *pgdA* gene into pVE3916, a pWV01 derivative (41, 42). A 1,276-bp DNA fragment was PCR amplified from the *E. faecalis* JH2-2 chromosome with primers LH47 and LH48 (see Table S1 in the supplemental material). The resulting fragment, including 173 bp upstream of the coding sequence of *pgdA* that contained the promoter, was digested with XhoI and HindIII restriction enzymes and cloned into pVE3916 similarly digested. The resulting plasmid (pVEF1843) was transformed into the *E. faecalis* Δ*pgdA* mutant. *E. faecalis* JH2-2 and its Δ*pgdA* derivative mutant were transformed with the empty pVE3916 plasmid.

***In vitro* deacetylation assays.** Chitooligosaccharides (Dextra Laboratories Ltd.; catalog numbers C8002, C8003, C8004, C8005, and C8006) were used at a concentration of 500 μM. Deacetylation assays were carried out for 6 h at 37°C using 10 mM phosphate buffer (pH 7.5) containing 250 μM $CoCl_2$ in a final reaction volume of 100 μl. Recombinant PgdA was added at a concentration of 15 μM, except for dose-response experiments (see Fig. 3). Reaction products were reduced as described below for peptidoglycan before mass spectrometry (MS) analyses.

Construction of JH2-2 derivative mutants. Isogenic mutants of *E. faecalis* JH2-2 were constructed by allelic exchange using the procedure previously described (24). The quadruple mutant Δ*oatA* Δ*sigV* Δ*dltA* Δ*pgdA* was constructed by introducing the Δ*pgdA* deletion in a Δ*oatA* Δ*sigV* Δ*dltA* background (30) using plasmid pgdMAD. Inducible expression of *sigV* under the control of nisin was carried out using plasmid pMSP3535-*sigV*, a pMSP3535 derivative (see Table S1 in the supplemental material).

Cell wall purification and peptidoglycan structural analysis. Peptidoglycan was purified from exponentially growing cells as described previously (14), freeze-dried, and resuspended in distilled water at a concentration of 20 mg/ml. Peptidoglycan (5 mg) was digested overnight with 250 μg of mutanolysin at 37°C in 500 μl of 20 mM sodium phosphate buffer (pH 6.0). Soluble disaccharide peptides were recovered in the supernatant following centrifugation (20,000 × *g* for 20 min at 25°C) and reduced with sodium borohydride as described previously (14). The reduced muropeptides were separated by reverse-phase high-performance liquid chromatography (rp-HPLC) on a C_{18} column (3-μm particles, 4.6 by 250 mm; Interchrom, Montluçon, France) at a flow rate of 0.5 ml/min. After 10 min in 10 mM ammonium phosphate, pH 5.6 (buffer A), muropeptides were eluted with a 270-min linear methanol gradient (0 to 30%) in buffer A. The peaks were analyzed by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) with a Voyager DE STR mass spectrometer (Applied Biosystems, Framingham, MA), using α-cyano-4-hydroxycinnamic acid as a matrix. For tandem mass spectrometry (MS-MS), data were collected with an electrospray time of flight mass spectrometer operating in the positive mode (Qstar Pulsar I; Applied Biosystems). The data were acquired with a capillary voltage of 5,200 V and a declustering potential of 20 V. The mass scan range was from *m/z* 350 to 1,500, and the scan cycle was 1 s.

PgdA protein production and purification. *E. faecalis* PgdA was overexpressed and purified as a recombinant protein with an N-terminal 6×His tag. *E. coli* BL21(DE3) cells harboring plasmid pQEF1843 were grown at 37°C in BHI broth. When the cultures had reached an optical density at 600 nm (OD_{600}) of 0.7, production of the recombinant proteins was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside, and incubation was continued for 4 h. The cells were harvested and resuspended in buffer A (50 mM Tris-HCl [pH 8.0] containing 300 mM NaCl), and crude lysates were obtained by sonication (five times for 30 s, 20% output; Branson Sonifier 450). Proteins were loaded onto Ni^{2+}-nitrilotriacetate agarose resin (Qiagen GmbH, Hilden, Germany), washed with 10 mM imidazole in buffer A, and eluted with 300 mM imidazole in buffer A. Recombinant His-tagged proteins were further purified by size exclusion chromatography on a Superdex75 HR 26/60 column (Amersham Biosciences, Uppsala, Sweden) equilibrated with a buffer containing 50 mM Na_2HPO_4, 150 mM NaCl, and 0.5 mM $CoCl_2$ (pH 7.5). The fractions were analyzed by sodium dodecyl sulfate-polyacrymamide gel electrophoresis (SDS-PAGE) and pooled (see Fig. S1 in the supplemental material). Protein concentration was estimated by measuring absorbance using a theoretical extinction coefficient of 41,370 M^{-1} cm^{-1} at 280 nm. Recombinant EF1843 protein was kept frozen at −80°C after addition of 25% (vol/vol) glycerol.

Western blotting. Immune rabbit serum against recombinant His-tagged EF1843 was generated by intramuscular immunization with the protein dialyzed against phosphate-buffered saline buffer at the GIP Plateforme Technologique d'Evreux (Evreux, France). For Western blot analyses, 10-ml samples of bacterial cultures were harvested at an OD_{600} of 0.5 by centrifugation. Cells were resuspended in 150 μl of 0.25 M Tris-HCl buffer (pH 7.5) and broken by vortexing for 3 min after addition of glass beads (0.1- to 0.25-mm diameter). Unbroken cells were removed by centrifugation (10 min at 6,000 × *g*, 4°C), and protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories). Crude extracts (10 μg) were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane using the Bio-Rad transblot system. The membranes were probed with anti-PgdA rabbit polyclonal serum at a 1/2,000 dilution. Western blots were developed using the enhanced chemiluminscence (ECL) detection kit (GE Healthcare, Little Chalfont, United Kingdom), according to the manufacturer's instructions.

Lysozyme sensitivity assay. Lysozyme sensitivity of the deletion mutants (see Fig. 4A) was assayed on LB agar supplemented with hen egg white lysozyme (Fluka, Buchs, Switzerland). Overnight cultures were harvested by centrifugation (5,000 × *g* for 5 min at room temperature),

washed once in saline water (0.9% [wt/vol] NaCl), and resuspended to an OD_{600} of 1. Two microliters of the 10^{-1}, 10^{-2}, and 10^{-3} dilutions was spotted on LB agar supplemented with 0.5 mg/ml or 0.75 mg/ml of lysozyme.

The contribution of PgdA to lysozyme resistance was monitored on gradient plates (see Fig. 4B) as follows: overnight cultures were diluted, and the cell suspension corresponding to 10^{-2} dilutions (ca. 10^6 CFU/ml) was streaked on a square petri dish containing a gradient of concentrations of nisin (up to 0.5 μg/ml) and lysozyme (up to 5 mg/ml) in opposite directions. The plates were photographed after 48 h of incubation at 37°C.

For semiquantitative assays (see Fig. S4 in the supplemental material), overnight cultures were prepared, and 2 μl each of the 10^{-1}, 10^{-2}, and 10^{-3} dilutions was spotted on LB agar containing 0.5 μg/ml of nisin and supplemented with different concentrations of lysozyme. The plates were photographed after 48 h of incubation at 37°C.

***G. mellonella* larva infection.** *G. mellonella* larvae were reared on bee's wax and pollen at 28°C in the dark. Larvae measuring 2.0 to 2.5 cm in length (body weight, 200 to 300 mg) with a cream-colored cuticle with minimal speckling or discoloration were used for each experiment. The *E. faecalis* strains used for infection were grown for 24 h in GM17. After centrifugation, cells were washed twice in 0.9% NaCl and resuspended to a final OD_{600} of 0.3. The size of the inoculums was confirmed by determining the number of CFU on solid GM17. Cohorts of 20 larvae were infected with 10 μl of a cell suspension through the left hindmost proleg into the hemocoel using a microinjector (KDS 100; KD Scientific) with a 1-ml syringe and 0.45- by 12-mm needles (Terumo). Five infected larvae were kept per petri dish, without food, at 37°C, and survival was monitored for 24 h by scoring every 2 h after 16 h of infection. As a negative control, the first and last cohort of infected larvae in every assay was sham infected with sterile 0.9% (wt/vol) NaCl solution. Larvae were considered dead when they displayed no movement in response to touch and had turned black.

Statistical analyses. Survival curves were analyzed with GraphPad Prism 5 using the log rank test.

RESULTS

EF1843 is not detected under laboratory conditions but is produced upon exposure to lysozyme. Previous work did not reveal any peptidoglycan structure modification associated with the deletion of *EF1843*, suggesting that the corresponding protein is not expressed under laboratory conditions, or that its activity was below detection thresholds. Western blot analyses indicated that the protein could not be detected in *E. faecalis* crude extracts when the cells were grown in rich medium (Fig. 1A, lane 1). In contrast, when the cells had been grown in the presence of lysozyme, a band corresponding to the expected protein size could be detected (Fig. 1A, lanes 2 to 4) and was absent in the Δ*EF1843* mutant extracts (Fig. 1A, lane 5). These results indicated that EF1843 protein is specifically produced upon exposure to lysozyme.

EF1843 is involved in peptidoglycan GlcNAc de-*N*-acetylation. Previous studies showed that *EF1843* deletion has no impact on *E. faecalis* peptidoglycan structure when the cells are grown in rich medium (24). To investigate EF1843-dependent peptidoglycan modifications, we used strain SAS (pMSP3535-*sigV*), a JH2-2 derivative with a nisin-inducible *sigV* copy (30). Nisin induction of *sigV* results in around a 2,000-fold increase in *EF1843* transcription compared to that with the control strain, SAS(pMSP3535), harboring the empty vector (30). After induction of EF1843 (Fig. 1B), peptidoglycan was purified and digested by mutanolysin, and the soluble muropeptides were reduced prior to rp-HPLC separation (Fig. 2). The muropeptide profile of the control strain, SAS(pMSP3535), harboring the empty vector (Fig. 2A), was virtually identical to the profile of the Δ*EF1843* mutant and to that of JH2-2 that had not been exposed to lysozyme (24). After induction of *sigV* and *EF1843* by nisin and lysozyme, several peaks appeared (Fig. 2B, peaks 1 to 8). MS analyses of these peaks revealed that they all contained muropeptides with *m/z* values matching those of deacetylated disaccharide-peptides (Fig. 2E). To further investigate the contribution of EF1843 to peptidoglycan deacetylation, *E. faecalis* JH2-2 and its isogenic Δ*EF1843* deletion mutant were grown in the presence of lysozyme, which we have shown to induce EF1843 production (Fig. 1C). The muropeptide profiles of the parental (Fig. 2C) and the mutant peptidoglycan (Fig. 2D) were overall very similar. However, a few peaks detected in the SAS(pMSP3535-*sigV*) muropeptide profile (Fig. 2B) were present in small amounts in the JH2-2 profile (labeled 1, 2, 3, and 5 in Fig. 2C) and absent from the Δ*EF1843* mutant profile (Fig. 2D). MS analysis confirmed the presence of deacetylated muropeptides in JH2-2 peaks 1, 2, 3, and 5, whereas no deacetylated muropeptides were detected in peak 1′, 2′, or 3′. The structure of the most abundant muropeptide corresponding to peak 3, matching the calculated mass of a disaccharide-pentapeptide with two alanyl residues as a lateral chain, was analyzed by electrospray tandem mass spectrometry (MS-MS) (Fig. 2F). Loss of a de-*N*-acetylated glucosamine residue (GlcN; −161.07) gave an ion at *m/z* 907.48. Additional loss of alanyl residues from the C terminus of the pentapeptide or the N terminus of the side chain gave additional ions characteristic of the muropeptides generated by mutanolysin (14). Together, our results therefore suggested that EF1843 is required for peptidoglycan GlcNAc de-*N*-acetylation.

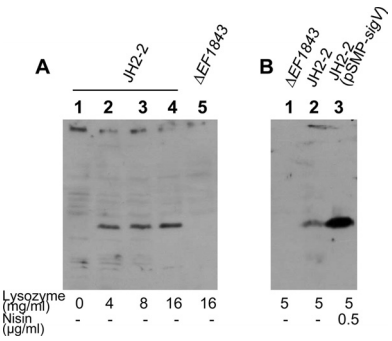
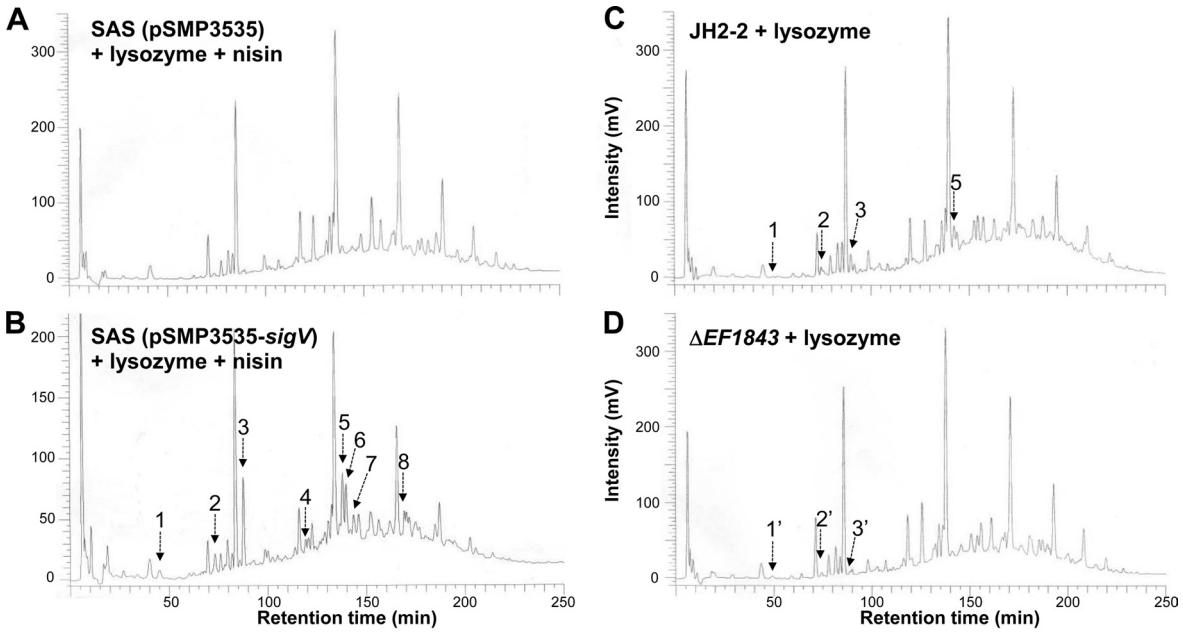


FIG 1 Immunodetection of EF1843 in *E. faecalis* cell extracts. (A) EF1843 detection by Western blotting after exposure to lysozyme. *E. faecalis* JH2-2 (lanes 1 to 4) and its isogenic Δ*EF1843* derivative (lane 5) were grown in M17 medium supplemented with glucose (GM17) to exponential phase before addition of various concentrations of lysozyme (0 to 16 mg/ml). After 1 h 30 min, the cells were harvested and crude extracts were probed with anti-EF1843 antibodies. (B) SigV-dependent overexpression of EF1843. The Δ*EF1843* mutant (lane 1), parental JH2-2 (lane 2), and JH2-2 harboring a nisin-inducible *sigV* gene (lane 3) were grown in GM17 to exponential phase before addition of lysozyme alone (5 mg/ml [lanes 1 and 2]) or in combination with nisin (0.5 μg/ml [lane 3]). After 1 h 30 min, the cells were harvested and crude extracts were probed with anti-EF1843 antibodies.

***In vitro* *N*-acetylglucosamine deacetylase activity of recombinant EF1843.** Chitooligosaccharides (2 to 6 residues) were used as substrates to assay EF1843 deacetylase activity *in vitro* (Fig. 3). Incubation of hexa-*N*-acetyl chitohexaose in the presence of EF1843 leads to the appearance of several ions with *m/z* values matching the loss of one to four acetyl groups (Fig. 3). Similarly, ions with *m/z* values matching deacetylation products were detected with the other chitooligosaccharides used as substrates, except for diacetyl chitobiose and GlcNAc (see Fig. S2 in the supple-



E

Peak	m/z [M+Na]+ [a] observed	m/z [M+Na]+ [a] theoretical	Inferred muropeptide	
1	948.43	948.46	DS-AQKAA	(-1 Ac)
2	948.33	948.46	DS-AQKAA	(-1 Ac)
3	1,090.46	1,090.52	DS-AQKAAAA	(-1 Ac)
4	1,968.87	1,968.95	DS-AQKAAA-DS-AQK	(-1 Ac)
5	2,110.70	2,111.02	DS-AQKAAA-DS-AQKAA	(-1 Ac)
6	2,110.92	2,111.02	DS-AQKAAA-DS-AQKAA	(-1 Ac)
7	2,069.17	2,069.01	DS-AQKAAA-DS-AQKAA	(-2Ac)
8	3,131.39	3,131.52	DS-AQKAAA-DS-AQKAAA-DS-AQKAAAA	(-1 Ac)
1'	ND [b]	NA [c]	NA	
2'	ND	NA	NA	
3'	ND	NA	NA	

[a] Sodiated molecular ions were the most abundant on MALDI-TOF mass spectra for all muropeptides.
[b] ND, not detected; [b] NA, not applicable

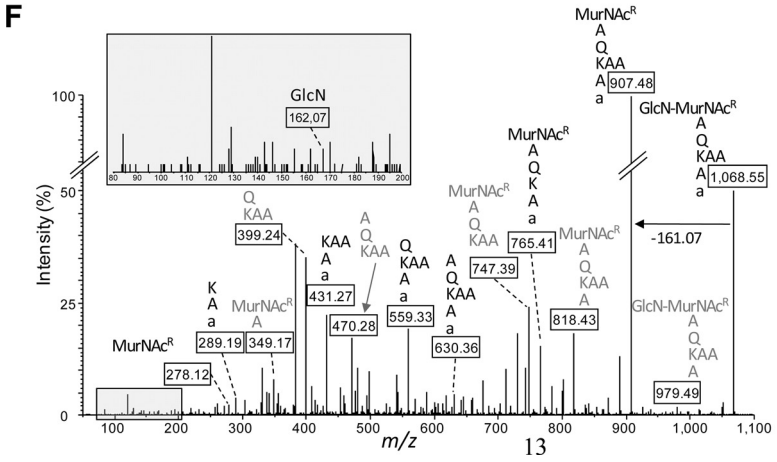


FIG 2 Detection of EF1843-mediated peptidoglycan modifications. EF1843 expression was induced by lysozyme and nisin as described for Fig. 1B. Peptidoglycan was extracted and digested by mutanolysin, and the muropeptides were reduced prior to separation by rp-HPLC. (A) rp-HPLC muropeptides from *E. faecalis* SAS(pMSP), a *sigV* mutant harboring the control vector pMSP3535, incubated with 0.5 μg of nisin and 5 mg/ml lysozyme. (B) rp-HPLC muropeptides from *E. faecalis* SAS(pMSP-*sigV*), a *sigV* mutant harboring a pMSP3535 derivative allowing inducible expression of *sigV*, incubated with 0.5 μg of nisin and 5 mg/ml lysozyme. Muropeptides in peaks 1 to 8 were analyzed by MS. Inferred structures are detailed in panel F. (C) rp-HPLC muropeptides from *E. faecalis* JH2-2 incubated with 5 mg/ml lysozyme. Muropeptides in peaks 1, 2, 3, and 5 were analyzed by MS. Inferred compositions are detailed in panel E. (D) rp-HPLC muropeptides from the *E. faecalis* Δ*EF1843* mutant incubated with 5 mg/ml lysozyme. Muropeptides in peaks 1′, 2′, and 3′ were analyzed by MS. (E) MALDI-TOF analyses of muropeptides contained in peaks 1 to 8. *m/z* values correspond to monoisotopic masses. DS, disaccharide; A, L-Ala or D-Ala; K, L-Lys; Q, D-*iso*-Gln; Ac, acetyl. (F) Electrospray MS-MS of muropeptide 3 from strain SAS(pMSP-*sigV*) incubated with lysozyme and nisin. The $[M+H]^+$ ion at *m/z* 1,068.55 was selected for fragmentation. The *m/z* values of the most informative ions are boxed, and the inferred structures are indicated. MurNAc^R, reduced MurNAc; GlcN, glucosamine; a, C-terminal D-Ala residue. Percentages on the ordinates show percentages of intensity.

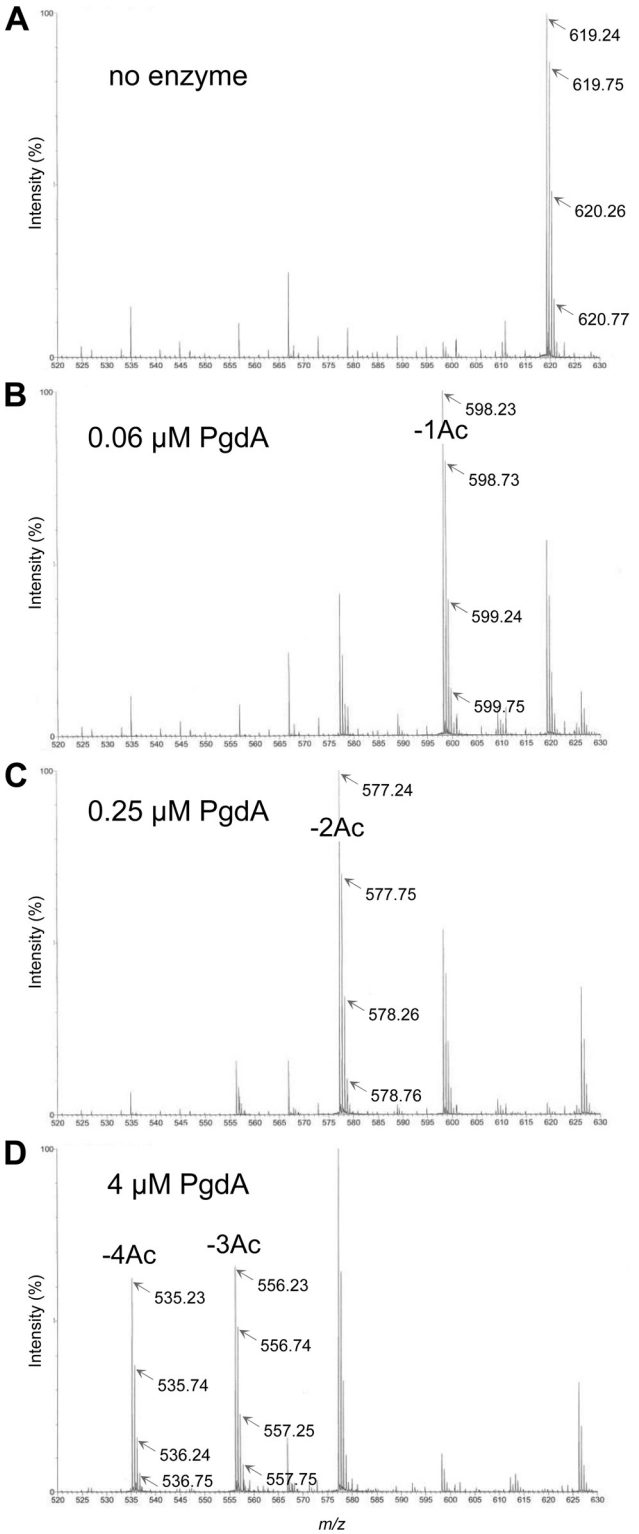


FIG 3 *In vitro* activity of recombinant EF1843. Deacetylase activity of recombinant EF1843 was assayed on hexaacetyl chitohexaose and analyzed by electrospray MS. The *m/z* values indicated correspond to $[M + 2H]^{2+}$ forms of the substrate and deacetylation products. (A) Mass spectrum of the hexaacetyl chitohexaose substrate. (B, C, and D) Mass spectra of the reaction products after incubation of the substrate with 0.06 μM, 0.25 μM, and 4 μM enzyme, respectively. *m/z* values of major ions matching deacetylation products are indicated together with the number of acetyl (Ac) groups missing.

mental material; other data not shown). Together, these results showed that EF1843 is an *N*-acetylglucosamine deacetylase, which we renamed PgdA.

PgdA-mediated peptidoglycan deacetylation inhibits lysozyme activity. Deletion of *pgdA* alone had no impact on lysozyme resistance level, with a MIC of >50 mg/ml, similar to that of the parental strain (24). Since previous studies revealed an additive contribution of several genes to lysozyme resistance in *E. faecalis* (30), *pgdA* deletion was combined with mutations in other genes involved in lysozyme resistance. Introduction of the *pgdA* deletion in a triple *oatA dltA sigV* mutant genetic background slightly decreased lysozyme resistance, indicating that peptidoglycan deacetylation contributes to protection of *E. faecalis* against lysozyme (Fig. 4A). However, the very limited contribution of PgdA to lysozyme resistance in a triple mutant background was expected in the absence of SigV that positively regulates *pgdA* expression. The contribution of PgdA to lysozyme resistance was therefore confirmed using strain SAS(pMSP3535-*sigV*), which allows massive overexpression of *pgdA* under the control of nisin (Fig. 1). For comparison purposes, we developed a gradient plate system, allowing fine-tuning of *pgdA* expression (using up to 0.5 μg/ml nisin), in conjunction with exposure to variable amounts of lysozyme (up to 5 mg/ml). Both the parental JH2-2 and *pgdA* strains grew normally in the presence of lysozyme at 5 mg/ml (Fig. 4B, lanes 1 and 6). In contrast, the growth of the *oatA dltA sigV* triple and the *oatA dltA sigV pgdA* quadruple mutants was inhibited by the lowest concentration of lysozyme used (Fig. 4B, lanes 2 and 3). Incorporation of nisin in the growth medium at a concentration that induces *sigV* expression (0.5 μg/ml) had no impact on cell viability or lysozyme resistance of the parental or *pgdA* strain (Fig. 4B, lanes 1 and 6). In the triple *oatA dltA sigV* mutant, nisin induction of *sigV* restored growth in the presence of the highest concentration of lysozyme used (Fig. 4B, lane 4). In contrast, upon induction of *sigV*, the nisin-dependent lysozyme resistance was strongly reduced in the quadruple *oatA dltA sigV pgdA* mutant (Fig. 4B, lane 5), demonstrating that the resistance to lysozyme conferred by SigV overexpression is mediated by PgdA. These results were confirmed by semiquantitative assays (30) using nisin (0.5 μg/ml) in combination with variable amounts of lysozyme (see Fig. S3A in the supplemental material).

To understand the mechanism by which PgdA mediates lysozyme resistance, we assayed the bactericidal effect of heat-denatured lysozyme. It has been shown previously that heat denaturation of lysozyme inactivates its catalytic activity while preserving its antimicrobial activity (25). Interestingly; gradient plate assays revealed that heat-denatured lysozyme was still highly active on the triple *oatA dltA sigV* mutant (Fig. 4C, lane 2), indicating that the catalytic activity of lysozyme against *E. faecalis* is not essential. Semiquantitative assays revealed that approximately twice as much heat-inactivated lysozyme is required to give growth inhibition similar to that given by native lysozyme (for example, compare 2-mg/ml and 4-mg/ml concentrations in Fig. S3A and S3B, respectively, in the supplemental material). In both gradient plates (Fig. 4C) and semiquantitative assays (Fig. S3B), the comparison of the triple *oatA dltA sigV* and the quadruple *oatA dltA sigV pgdA* mutants showed that PgdA had only a marginal effect on the antimicrobial peptide bactericidal effect of lysozyme. We therefore concluded that peptidoglycan deacetylation by PgdA inhibited both lysozyme catalytic activity and, to a lesser extent, lysozyme antimicrobial peptide activity. *pgdA* deletion had no clear impact

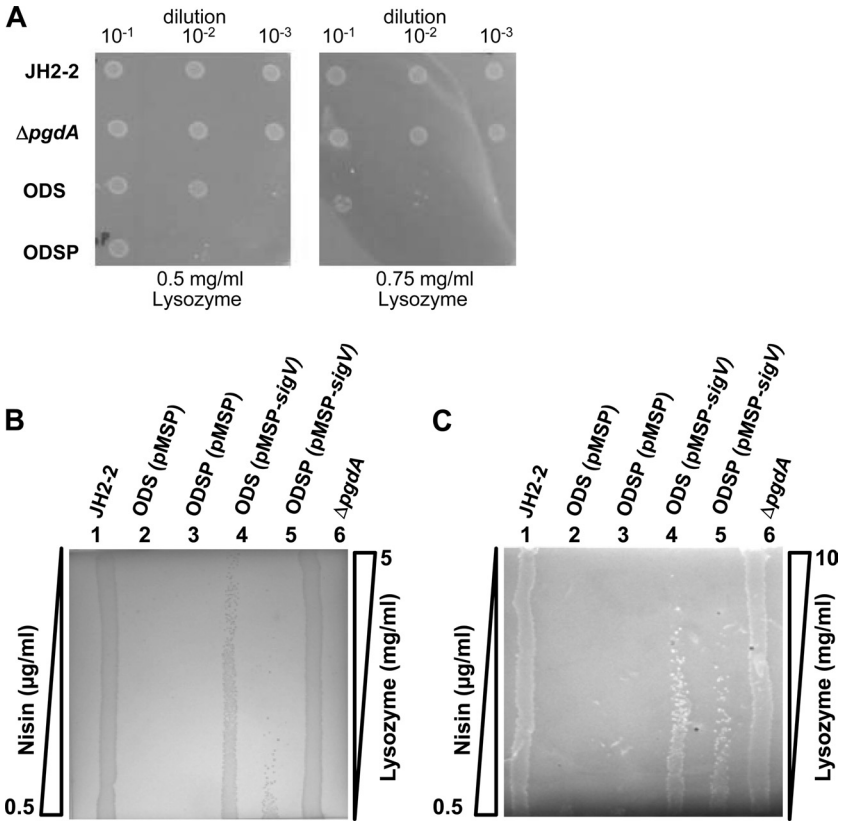


FIG 4 Contribution of PgdA activity to lysozyme resistance. Overnight cultures were washed and resuspended in saline solution at an OD_{600} of 1. Cells were grown on LB agar medium supplemented with lysozyme alone or in combination with nisin to induce *sigV*-dependent PgdA overexpression. Growth was monitored after 48 h at 37°C. (A) Two microliters corresponding to 10-fold serial dilutions (10^{-1}, 10^{-2}, and 10^{-3}) were spotted on LB medium supplemented with 0.5 mg/ml or 0.75 mg/ml of lysozyme. ODS, *oatA dltA sigV*; ODSP, *oatA dltA sigV pgdA*. (B) Cell suspensions corresponding to 10^{-2} dilutions were applied on a square petri dish containing concentration gradients of lysozyme (up to 5 mg/ml) and nisin (up to 0.5 μg/ml). Lane 1, JH2-2; lane 2, *oatA dltA sigV* mutant (pMSP); lane 3, *oatA dltA sigV pgdA* mutant (pMSP); lane 4, *oatA dltA sigV* mutant (pMSP-*sigV*); lane 5, *oatA dltA sigV pgdA* mutant (pMSP-*sigV*); lane 6, *pgdA* mutant. (C) The same samples as in panel B were applied to a square petri dish containing a concentration gradient of heat-inactivated lysozyme (60 min at 100°C; up to 10 mg/ml) and nisin (up to 0.5 μg/ml).

on the bactericidal activity of three other cationic antimicrobial peptides: pexiganan (21), polymyxin B, and nisin (see Fig. S4 in the supplemental material).

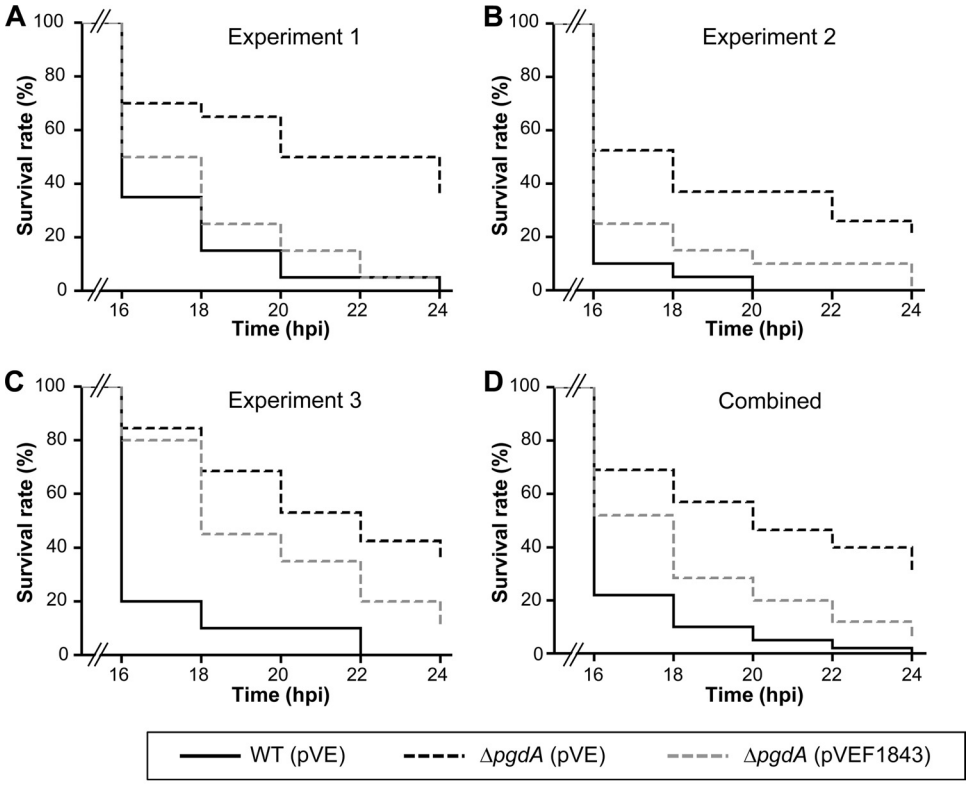
PgdA contributes to *E. faecalis* virulence. We used *G. mellonella* as an animal model (20, 28, 31, 32) to compare virulence of the parental JH2-2 strain, its isogenic *pgdA* mutant, and the complemented mutant. Three independent experiments were carried out using JH2-2 and the *pgdA* deletion mutant, both harboring the empty vector used for complementation, as well as the complemented *pgdA* mutant (Fig. 5A to C). In the combined results (Fig. 5D), significant differences in mortality rates were noted between the parental and mutant strains (log rank test; $P < 0.0001$). As expected, complementation of the *pgdA* deletion partially restored mortality rates comparable to those of the parental strain (Fig. 5D), and in the control group infected with sterile saline solution, no larvae died in any of the replicates (data not shown). These results therefore showed that *pgdA* contributes to the virulence of *E. faecalis*.

DISCUSSION

Based on sequence homology, EF1843 was previously identified as a putative peptidoglycan deacetylase in *E. faecalis* (24). However, peptidoglycan deacetylation modification has not been reported so far for *E. faecalis* (15, 24), and previous studies did not reveal any contribution of EF1843 to peptidoglycan structure under laboratory conditions. Our objectives were to identify the role of *E. faecalis EF1843* in resistance to lysozyme and to test its contribution to the virulence of this opportunistic pathogen.

EF1843 transcription is regulated by σ^V, an ECF sigma factor involved in lysozyme resistance (3, 30). Our results revealed that *E. faecalis* peptidoglycan deacetylation occurs after exposure to lysozyme, suggesting that EF1843 is an effector of the σ^V signaling cascade induced by lysozyme (30). We showed that lysozyme triggers the production of EF1843 and that, in turn, this protein deacetylates GlcNAc residues in peptidoglycan. This mechanism, which allows *E. faecalis* to sense one of the components of the host innate immune response, is reminiscent of that described for *Streptococcus suis* (18). In *S. suis*, peptidoglycan de-*N*-acetylation is extremely low when the cells are grown in rich medium. Although the host signal(s) inducing peptidoglycan de-*N*-acetylation has not been identified, the expression of *S. suis* deacetylase is strongly stimulated *in vivo* (18).

Both *E. faecalis* and *S. aureus* can tolerate very high lysozyme concentrations (up to 50 mg/ml) compared to other Gram-positive bacteria such as *Listeria monocytogenes* (6) or *Streptococcus pneumoniae* (43). These high resistance levels rely on two main



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	P values			
	Exp. 1	**Exp. 2**	**Exp. 3**	**Combined**
JH2-2 (pVE) *vs* Δ*pgdA*(pVE)	0.0003	0.001	<0.0001	<0.0001
Δ*pgdA*(pVE1843) vs Δ*pgdA*(pVE)	0.0039	0.0285	>0.05	<0.0001
JH2-2 (pVE) *vs* D*pgdA*(pVEF1843)	>0.05	>0.05	0.0009	0.0005

FIG 5 Survival of *G. mellonella* inoculated with *E. faecalis* JH2-2 or *pgdA* derivatives. Wax worm larvae were inoculated with 1.9×10^6 CFU of the parental (WT) JH2-2 strain transformed with the empty pVE3916 plasmid [JH2-2 (pVE); solid line]; 2.1×10^6 CFU of the isogenic *pgdA* mutant strain transformed with the empty pVE3916 plasmid [Δ*pgdA* (pVE); black dotted line]; or 1.8×10^6 CFU of the *pgdA* mutant strain transformed with pVE3916 harboring a functional *pgdA* gene [Δ*pgdA* (pVEF1843); gray dotted line]. Survival was monitored between 16 and 24 h postinfection (hpi) at 37°C using 20 larvae per dose per strain per experiment. Three independent experiments (A, B, and C) and the combined results (D) are shown. (E) *P* values for pairwise comparisons. No deaths occurred in larvae injected with sterile saline solution as a control (not shown).

mechanisms: (i) peptidoglycan *O*-acetylation mediated by *oatA* and (ii) modification of the negative net charge of the bacterial cell envelope through addition of D-alanine residues on teichoic and lipoteichoic acids, mediated by *dlt* genes (30). Whereas *oatA* inactivation has similar effects on the MIC of lysozyme in both *E. faecalis* and *S. aureus* (ca. 5-fold decrease), *dltA* inactivation has a more pronounced effect in *S. aureus* than in *E. faecalis* (25-fold decrease in lysozyme MIC versus 5-fold, respectively). Interestingly, *oatA* and *dltA* mutations have a synergistic effect in *S. aureus* (10,000-fold reduction of the MIC) and at best an additive effect in *E. faecalis* (25-fold reduction of the MIC). Previous studies have shown that *S. aureus oatA* and *dltA* are part of the *graRS* regulon, which includes 248 genes (25). It is therefore tempting to assume that in *S. aureus*, the synergy between *oatA* and *dltA* results from other mechanisms triggered by a complex feedback control involving *graRS*. In *E. faecalis*, the key transcriptional regulator orchestrating lysozyme resistance is σ^V. *E. faecalis* σ^V does not regulate the expression of *dltA* or *oatA* (30), contrary to *B. subtilis* σ^V (22, 26). As a consequence, high-level resistance to lysozyme in *E. faecalis* is achieved through three distinct pathways leading to cell wall modifications (involving *sigV*, *oatA*, and *dltA*).

Interestingly, we showed that *E. faecalis* harboring simultaneous deletions of *oatA*, *dltA*, *sigV*, and *pgdA* still displays resistance to lysozyme (Fig. 4A). Previous studies showed that addition of L-lysine on phosphatidylglycerol by the multiple peptide resistance factor (MprF) described for *S. aureus* (16) does not contribute to lysozyme resistance in *E. faecalis* (30). Moreover, no homolog of the lysozyme inhibitors found in Gram-negative (1, 8, 39, 40) or Gram-positive (5) bacteria is found in the *E. faecalis* genome. Therefore, other mechanisms responsible for the unusual resistance to lysozyme are yet to be identified in *E. faecalis*.

Peptidoglycan deacetylation has been reported as a virulence factor in a number of important pathogens such as *Listeria monocytogenes* (2, 35), *S. pneumoniae* (12), *S. suis* (18), and

S. aureus (4). In this study, we also showed its involvement in *E. faecalis* virulence in an insect infection model. In conclusion, *E. faecalis* appears to have evolved one of the most complex combinations of lysozyme resistance mechanisms among Gram-positive bacteria. This unique arsenal ensures the capacity of this organism to thrive inside mammalian hosts, both as a commensal and as a pathogen.

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